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The CatSper channel: a polymodal chemosensor in human sperm

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1st Editorial Decision 16 September 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see, while all three referees consider the study as very interesting in principle, it becomes clear that the depth of analysis is not yet sufficient. In particular, and as detailed by referee 1 (and 2) stronger causal evidence is needed that GPCRs are not involved. Also, deeper analysis of the effect of extracellular nucleotides will be needed as mentioned by all three referees. Now, we recognise that referee 1 is more demanding. Still, on balance and after looking into the case in depth, we have come to the conclusion that we will be able to consider a suitably revised manuscript that addresses the referees' concerns, but that the points put forward by referee 1 need to be addressed by further experimentation to his/her full satisfaction. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version and on the final assessment by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as

soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

This is a follow up study by the authors who reported that progesterone increases sperm Ca2+ by activation of CatSper in a GPCR-independent manner. However, how progesterone activates CatSper was not resolved by the previous of present study. In the present study, the authors tested effect of odorants and other compounds on sperm Ca2+ to come to two significan conclusions: a) Odorant, cNMP and other compounds act in a GPCRs- independent manner and b) All compounds activate CatSper to increase sperm Ca2+. Although both conclusions are of interest, none is supported by sufficiently strong results.

1. The only evidence for a lack of involvement of GPCRs is the kinetic of Ca2+ activation. This is not sufficient by any means. One example, activation of TRP channels in the drosophila eye occurs much faster that what the authors recorded in sperm. With the incredible small volume/surface in sperm everything will occur fast. The authors must exclude involvement of GPCRs more rigorously. Just two examples, show that inhibition of PLC by U-73122, and more importantly, inhibition of GPCRs-mediated signaling by infusing the sperm with GDPbetaS through the patch pipette does not prevent activation of CatSper by all the compounds in Fig. 7.

2. The Ca2+ signal has two components, only one is inhibited by the semi-selective CatSper inhibitors used. What pathway mediates the secondary Ca2+ increase? If both phases are mediated by CatSper, is the late Ca2+ increase activated by an indirect pathway. This should be addressed by direct experiments.

3. The conclusion in page 8, end of last paragraph that the agonists tested increase cAMP independent of GPCRs is based on the single observation that IBMX does not augment the increase in cAMP. This is far from being sufficient for the conclusion. Note that use of AC inhibitors by the Hatt group case to opposite conclusion. Similar approach should be applied in the present work. At a minimum the discrepancy between the studies need to be explained by experiments designed to explain why the inhibitor of particulate AC SQ22536 had the effects reported in human sperm.

4. The studies with the cNMP are interesting, but not complete. It is very unlikely that they are the native ligand activating CatSper. However, they indicate that CatSper may be activated by nucleotide. Considering that CatSper is activated by extracellular nucleotides, it will thus be important to test whether low concentrations of ATP, UTP and nucleotides that do not activate P2X2R can activate CatSper. In this respect, it will be informative to first determine if human sperm expresses active P2X2R, as was reported recently for mouse sperm (Clapham et al, PNAS), and then isolate potential activation of CatSper by ATP.

5. For the findings in Fig. 7 to be of the significance attributed to them the authors have to show that the maximal concentration of each agonist used actually activate CatSper current. In addition, to complete the Figure, the effect of Angiotensin, calcitonin and FPP on CatSper current and the Ca2+ dose response should be included.

Referee #2

Goodwin et al. reported in this manuscript two very interesting and novel findings. First, they discovered that CatSper, a sperm-specific Ca2+ channel absolutely required for sperm function and male fertility, is the target of several well-known "sperm-activating" molecules including odorants and 8-Br-cGMP. Odorants and cyclic nucleotides have been known to elicit Ca2+ changes in sperm but the targets have been, as now shown by authors, perhaps wrongly assigned in the past. Second and most interestingly, the authors found that odorants and 8-Br-cGMP both activate CatSper via extracellular space, and, in the case of odorant, an intracellular increase of cAMP/cGMP concentration doesn't seem to be involved. These findings are quite unexpected and provocative. The studies are of high interest to those in the fields of reproductive biology, ion channel and Ca2+ signaling. The manuscript is well written and I believe is suitable for publishing in the Journal after some minor changes.

I have only a few suggestions.

1) Throughout the paper, the authors use the cell-permeable cAMP/cGMP analogs; it's not clear whether the authors also suggest that cyclic nucleotides (cGMP/cAMP) activate CatSper and lead to [Ca2+]i increase via an extracellular mechanism. If so, this appears to be counterintuitive as it is unlikely that the extracellular cGMP/cAMP concentration would have significant change under physiological conditions. In the patch-clamp recording that shows 8-Br-cGMP activates CatSper from outside, have the authors tested cGMP that's not cell permeable. In addition, earlier work from the same authors (e.g. Wiesner et al. (1998) JCB 142: 473-484) using local UV uncaging in bovine sperm suggest that the action of cGMP in [Ca2+]i changes is likely through an intracellular action. The authors should clarify these apparently conflicting/confusing results in the discussion session.

2) The finding that odorants don't act on GPCRs and via intracellular messengers, as commonly believed, is quite intriguing and it directly challenges the dogma. There are two major pieces of evidence supporting the authors' conclusion. First, the time delay between the addition of the ligand and the increase of [Ca2+]i is < 36 ms (the limitation of the measurement), too short for a "conventional" GPCR pathway. However, process involving GPCRs and intracellular signaling with this short time delay, although uncommon, is not impossible. An example would be the Drosophila photoreceptors. It's possible that the geometry of sperm cell (<1 micron in the tail, way smaller than most other cells) makes it feasible for an ultra fast signaling pathway. Second, the authors detected no cAMP concentration increase when sperm were stimulated with the ligands. Again, there could be alternative explanation for this observation. One possibility is that the increase is local (e.g. restricted to the principal piece where CatSper is localized) and transient. This kind of local and transient change would escape the detection. We perhaps have seen such an example in sperm with the sNHE (SLC9A10) KO. sNHE almost certainly affects sperm pH dynamics, but there is no obvious detectable global pHi difference between WT and the KO sperm, presumably because the pHi detection methods used could not detect localized changes in sperm principal piece where sNHE protein is found.

The authors need to discuss the above possibilities. Even better, using patch clamp recordings with GTPγS or GDPβS in the pipette, the authors could significantly strengthen their conclusion, a major one that, if proven, would drastically change how we think about the signaling pathway,

Additional minor suggestions:

3) The Y-axes in several figures (Figs. 1, 3, 5, 7, S1, S4 & S7) should be changed to "ΔF/F0" from "ΔF/F". To make it easier for the general audience to understand, explain DF/F0 when first used (e.g. Fig. 1 legend).

4) In the abstract, consider changing "binding to an extracellular site" to "acting on an extracellular site" because, strictly speaking, a binding is not demonstrated in the studies.

5) page 14, the method section, the solutions used in the patch clamp experiments seem to have a

iquid junction potential of ~15 mV, depending on the configuration. Was the potential corrected in the figures and in the Vrev calculation?

6) in figures where currents from step protocols are shown (Fig. 2A, C; Fig. 6 A, C, E), there appear to be large capacitance currents that could confuse "non-expert" readers. Consider applying a low-pass filter and stating the filter frequency in the method section.

7) figure 2, how easily is the action of bourgeonal reversible? it would be nice to include a washout trace in the figure.

8) Figure S2 appears to be inappropriately cited in page 4. Please clarify the conclusion of this figure.

Referee #3

This is a very interesting comprehensive study with data that are novel and have potentially wide ranging impact. The authors have expertise in the area of cell signalling in mammalian and invertebrate spermatozoa and have very recently characterised CatSper as a primary channel activated by progesterone in human spermatozoa. This has had a significant impact on the field and promises to be a very exciting area for research. Recently for example Navarro and colleagues have examined role of P2X2 receptors and sperm function using patch clamping (PNAS 2011 108(34):14342-7) and our understanding of this field is likely to grow exponentially.

In this series of experiments the authors extend their observations examining the range of agonists that can activate CatSper using fluorimetry and patch clamping. Very surprisingly they report that CatSper is activated by a number - and range - of small molecules fascinatingly suggesting considerable promiscuity. Particularly interesting is the concept that cGMP/cAMP activate CatSper outside the cell rather than from inside. The authors show that odorants activate CatSper and discuss in detail the discrepancies regarding membrane AC.

Questions/queries

1. Why was only 4mM bicarbonate used for cAMP assessments?
2. An explanation is required why CatSper (no currents activated) is not activated by calcium (page 6).
3. There is considerable reliance on use of drugs e.g. NNC 55-0396 which are not CatSper specific hence other effects of these drugs needs to be strictly controlled/eliminated.
4. Importantly, the physiological context needs explaining. An explanation/speculation of how the cell may operate in the female tract in a 'soup' of chemicals for a number of days - how is CatSper likely to respond/activate/deactivate? Progesterone, prostaglandins etc are present at the same time in the tract - how does CatSper respond to a combination of reported agonists? What is the explanation for figure 1G?
5. cAMP is used as a chemo attractant e.g. in Dictyostelium - is this a possible mechanism in humans?
6. With cAMP assessments as an endpoint the authors report no generation of cAMP with reportedly known agonists. What is the reason for the discrepancy in the results with other previous studies? Is it species differences and/or technique? The n values need clarification in Fig 4.
7. In Supplementary Fig 2 - live AR needs to be clarified e.g. in title.

1st Revision - authors' response 13 December 2011

(see next page)

General response to all referees

In the abstract and the introduction, we rephrased some sentences to improve readability without changing the meaning. The discussion section was also amended to include discussion of new data. The request of referees for additional experiments required major changes in the results section to incorporate new results. We give an overview of these changes here and provide a detailed account in the respective responses to the referees' comments.

1. The first two chapters describing the action of odorants on sperm´s $[Ca^{2+}]_i$ and on CatSper currents were largely unchanged. In the first paragraph of the third chapter, we focus on the action of bourgeonal on cAMP levels in sperm bathed in high and low bicarbonate.

We also include additional data on the effect of the tmAC inhibitors MDL12330A and SQ22536 on resting cAMP levels and on the cAMP increase evoked by adenosine. We include additional data concerning the action of calcitonine, FPP, angiotensin, and adenosine on $[Ca^{2+}]_i$.

Next, we present new results concerning the pharmacology of SQ22536, MDL12330A, and U73122 probed by Ca^{2+} fluorimetry and electrophysiology. Finally, we present the kinetic Ca^{2+} fluorimetry and, moreover, additional patch-clamp recordings in the presence of GDPβS.

We left the chapters concerning the action of cyclic nucleotides on CatSper largely unchanged. We only included additional data concerning the action of cNMPs and ATP on $[Ca^{2+}]_i$ and the action of ATP on membrane currents.

We moved the data concerning the action of menthol to the results section and moved the former Figure 7 (now Figure 9), which depicts the dose-response curves for various CatSper agonists, to the discussion section.

The discussion section includes now a new paragraph discussing the pitfalls of pharmacological experiments in sperm.

Response to referee #1:

1. The only evidence for a lack of involvement of GPCRs is the kinetic of Ca^{2+} activation. This is not sufficient by any means. One example, activation of TRP channels in the drosophila eye occurs much faster that what the authors recorded in sperm. With the incredible small volume/surface in sperm everything will occur fast. The authors must exclude involvement of GPCRs more rigorously. Just two examples, show that inhibition of PLC by U-73122, and more importantly, inhibition of GPCRs-mediated signaling by infusing the sperm with GDPbetaS through the patch pipette does not prevent activation of CatSper by all the compounds in Fig. 7.

Olfactory neurons, vertebrate photoreceptors, and sea urchin sperm respond to stimulation with a latency of ~ 50- 300 ms. In these cells, signalling occurs in cilia or flagella that have the same small volume-to-surface ratio. *Drosophila* photoreceptors is a special case; signalling occurs in microvilli – even much smaller than subcellular compartments – and is organised in transducisomes. Thus, we maintain that a latency of < 36 ms - if any - for the Ca^{2+} responses in sperm is difficult to reconcile with an olfactory-like, metabotropic pathway. Moreover, membrane currents were recorded without ATP and GTP in the pipette to preclude G-protein signalling. We mentioned this now in the main text. Notwithstanding, we followed the suggestion of the referee and used the drugs U73122 and GDPβS. Unfortunately, U73122 is not suitable, even dangerous, to study Ca^{2+} signalling in human sperm: Micromolar concentrations (~ 10 μM) have been used to interfere with PLC activity (see for example Bahat and Eisenbach 2010). However, at nanomolar concentrations, the drug evokes pronounced Ca^{2+} signals on its own (Fig. 4 and Supplementary Fig. S7). This prevents to test whether the drug inhibits Ca^{2+} signals evoked by CatSper agonists. Presumably, U73122, a steroid derivative, directly activates CatSper like progesterone. Odorants, progesterone, prostaglandins, and 8-Br-cGMP potentiated CatSper currents even in the presence of GDPβS (Fig. 5 and Supplementary Fig. S8), strengthening our conclusion that GPCRs are not involved.

2. The Ca^{2+} signal has two components, only one is inhibited by the semi-selective CatSper inhibitors used. What pathway mediates the secondary Ca^{2+} increase? If both phases are mediated by CatSper, is the late Ca^{2+} increase activated by an indirect pathway. This should be addressed by direct experiments.

It has been shown by us and by others (e.g. Spehr et al. 2003, Veitinger et al. 2011) that the odorant-induced Ca^{2+} signals rest on Ca^{2+} influx. We only state that the Ca^{2+} influx is mediated by CatSper. The sustained elevation of $[Ca^{2+}]_i$ might reflect Ca^{2+} release from intracellular stores triggered by the Ca^{2+} influx via CatSper (see Bedu-Addo et al. 2008). Alternatively,

bourgeonal might also inhibit Ca^{2+} export mechanisms, which could account for the sustained Ca^{2+} component. In electrophysiology, mibefradil inhibited the odorant-induced currents completely. Thus, inhibition of CatSper does not reveal another conductance or pathway activated by odorants. A potential Ca^{2+}-induced Ca^{2+} release or inhibition of Ca^{2+} transport is beyond the scope of the present manuscript and should be addressed in future studies.

On a final note, the CatSper inhibitors NNC and mibefradil – as well as many other drugs/inhibitors employed - display a complex behaviour. Because the CatSper inhibitors evoke Ca^{2+} signals on their own at concentrations > 10 µM - 30 µM, it is prohibitive to test, whether higher drug concentrations completely abolish Ca^{2+} signals. Therefore, we caution against mechanistic speculations. However, in electrophysiology, NNC and mibefradil completely block currents and the interpretation of their actions is straightforward.

3. The conclusion in page 8, end of last paragraph that the agonists tested increase cAMP independent of GPCRs is based on the single observation that IBMX does not augment the increase in cAMP. This is far from being sufficient for the conclusion. Note that use of AC inhibitors by the Hatt group came to opposite conclusion. Similar approach should be applied in the present work. At a minimum the discrepancy between the studies need to be explained by experiments designed to explain why the inhibitor of particulate AC SQ22536 had the effects reported in human sperm.

The Hatt/Spehr model of an olfactory cAMP-signalling pathway in human sperm makes two predictions: (1) Bourgeonal should increase cAMP levels; and (2) cAMP should increase $[Ca^{2+}]_i$. Unfortunately, our data and data from other groups (even from Hatt/Spehr) do not support these predictions: Bourgeonal does not elevate cAMP (Figure 3; see also a new report from this group Veitinger et al. 2011) and cAMP does not elevate $[Ca^{2+}]_i$ in mouse and human sperm (Wennemuth et al. 2003, Carlson et al. 2007, Strünker et al. 2011). Instead, previous work on the action of odorants solely relied on pharmacological experiments using tmAC inhibitors. At the request of the referee, we have examined the pharmacology of these drugs.

First, we tested whether inhibitors of tmACs (MDL12330A and SQ22936) affect cAMP levels in human sperm (Figure 5B). Both drugs did not lower resting cAMP – contrary to what is expected from tmAC inhibitors. Moreover, the drugs failed to inhibit the adenosine-evoked increase of cAMP. These results support the notion that adenosine does not act via GPCRs and tmACs. In mouse sperm, the inhibitors also fail to inhibit the adenosine action (Schuh et al. 2006). Instead, we now unveil that SQ22936 (but not MDL12330A) elevates cAMP levels in human sperm (Figure 5B). A similar action of SQ22936 can be inferred from the effect of the drug on the flagellar beat in mouse sperm (Schuh et al. 2006). We suspect that SQ22936, at

these high concentrations, inhibits PDE activity and thereby elevates cAMP levels. Taken together, SQ22936 is not a suitable tool to interfere with tmAC signalling in mammalian sperm. Second, Veitinger et al. 2011 reported that 200 μM SQ22936 completely inhibited odorant-induced Ca^{2+} signals. However, we failed to reproduce these results: Ca^{2+} signals were similar in the absence and presence of the drug (Figure 4). Moreover, we noticed that the Hatt/Spehr group published contradictory results on several occasions: In Veitinger et al. 2011, Ca^{2+} signals were completely inhibited by 200 μM SQ22936; in Spehr et al. 2004, Ca^{2+} signals were identical in the absence and presence of 200 μM SQ22936 and concentrations of 3 -10 mM were required to inhibit odorant-induced Ca^{2+} signals (see their Fig. 4). We caution against results obtained with high millimolar concentrations of small molecule inhibitors. At such high concentrations, drugs can hardly be considered as "specific".

Third, we tested whether MDL12330A inhibits the odorant-induced Ca^{2+} signals (Figure 4). In contrast to SQ22936, the drug suppresses the Ca^{2+} signals, confirming the results by Veitinger et al. 2011 and Spehr et al. 2003. However, the drug also abolishes progesterone- and alkaline-evoked Ca^{2+} signals and CatSper currents, demonstrating that the drug, in fact, inhibits CatSper channels (Figure 4). This result doesn't come as a surprise. MDL12330A has been known for almost 20 years as an inhibitor of various Ca^{2+} channels. In conclusion, we show that U73122, SQ22936, and MDL12330A are unsuitable to probe GPCR, PLC, and tmAC signalling in human sperm. They are non-specific and cause cellular responses unrelated to their original targets, which makes interpretations of results difficult, if not impossible. We are grateful to the referee for suggesting these experiments. It is a lesson on the fallacy of pharmacological tools.

4. The studies with the cNMP are interesting, but not complete. It is very unlikely that they are the native ligand activating CatSper. However, they indicate that CatSper may be activated by nucleotide. Considering that CatSper is activated by extracellular nucleotides, it will thus be important to test whether low concentrations of ATP, UTP and nucleotides that do not activate P2X2R can activate CatSper. In this respect, it will be informative to first determine if human sperm expresses active P2X2R, as was reported recently for mouse sperm (Clapham et al, PNAS), and then isolate potential activation of CatSper by ATP.

This remark is well taken. We performed the requested experiments. We now include data (Figure 7) illustrating that very high physiological concentrations of cNMPs (~ 1 mM) do not evoke Ca^{2+} signals in human sperm. We also tested whether human sperm express functional P2X2 receptors. ATP (1 mM) did not evoke Ca^{2+} signals in human sperm (Figure 7). Moreover, under similar conditions used in Navarro et al. 2011, ATP did not stimulate membrane currents in human sperm (Figure 7). We conclude that CatSper is insensitive to ATP and that human sperm do not express functional P2X2 receptors.

We agree that the activation of CatSper by 8-Br-cNMPs might be an artefact of using high concentrations. However, it points to a potential source of misleading interpretations and highlights the promiscuity of CatSper.

5. For the findings in Fig. 7 to be of the significance attributed to them the authors have to show that the maximal concentration of each agonist used actually activate CatSper current. In addition, to complete the Figure, the effect of Angiotensin, calcitonin and FPP on CatSper current and the Ca2+ dose response should be included.

We agree that, in principle, the fluorimetry results should be corroborated with patch-clamp experiments. We mention this cautionary remark in the discussion.

We did not claim that angiotensin, calcitonin, and FPP activate CatSper channels. In fact, none of these peptides evoke Ca^{2+} signals at physiological concentrations (Supplementary Figure 6). The physiological function of these ligands in sperm is obscure, considering that they neither elevate $[Ca^{2+}]_i$ nor cAMP levels in human sperm.

On a quite general note, we recognize that our results question more than 20 years of research on cAMP signalling in mammalian sperm. Moreover, we provide evidence that previous conclusions and models were based on artefactual pharmacological actions. Finally, we also realize that we cannot reproduce some previous reports.

Response to referee #2:

1. Throughout the paper, the authors use the cell-permeable cAMP/cGMP analogs; it's not clear whether the authors also suggest that cyclic nucleotides (cGMP/cAMP) activate CatSper and lead to [Ca2+]i increase via an extracellular mechanism. If so, this appears to be counterintuitive as it is unlikely that the extracellular cGMP/cAMP concentration would have significant change under physiological conditions. In the patch-clamp recording that shows 8-Br-cGMP activates CatSper from outside, have the authors tested cGMP that's not cell permeable. In addition, earlier work from the same authors (e.g. Wiesner et al. (1998) JCB 142: 473-484) using local UV uncaging in bovine sperm suggest that the action of cGMP in [Ca2+]i changes is likely through an intracellular action. The authors should clarify these apparently conflicting/confusing results in the discussion session.

Concerning the action of extracellular cNMPs, see the response to referee #1. We did not suggest that extracellular cNMPs activate CatSper; in fact, they don't as we show.

We now discuss previous work including that by Wiesner et al. 1998. There is a growing body of evidence that considerable differences exist between mammalian sperm species. HVCN1 currents, activation of CatSper by progesterone, expression of purinergic receptors P2X2, and KSper currents - to name a few clear-cut cases - differ between mouse and human sperm. In fact, with respect to ionic currents, there seem to be more differences rather than similarities between mouse and human sperm. Therefore, it can not be excluded that bovine sperm express ion channels that are activated by intracellular cNMPs. This should be addressed by patch-clamp recordings from bovine sperm. However, mouse and human sperm are lacking such channels.

2. The finding that odorants don't act on GPCRs and via intracellular messengers, as commonly believed, is quite intriguing and it directly challenges the dogma. There are two major pieces of evidence supporting the authors' conclusion. First, the time delay between the addition of the ligand and the increase of [Ca2+]i is < 36 ms (the limitation of the measurement), too short for a "conventional" GPCR pathway. However, process involving GPCRs and intracellular signaling with this short time delay, although uncommon, is not impossible. An example would be the Drosophila photoreceptors. It's possible that the geometry of sperm cell (<1 micron in the tail, way smaller than most other cells) makes it feasible for an ultra fast signaling pathway. Second, the authors detected no cAMP concentration increase when sperm were stimulated with the ligands. Again, there could be alternative explanation for this observation. One possibility is that the increase is local (e.g. restricted to the principal piece where CatSper is localized) and transient. This kind of local and transient change would escape the detection. We perhaps have seen such an example in sperm with the sNHE (SLC9A10) KO. sNHE almost certainly affects sperm pH dynamics, but there is no obvious detectable global pHi difference between WT and the KO sperm, presumably because the pHi detection methods used could not detect localized changes in sperm principal piece where sNHE protein is found.

Concerning the latency of the Ca^{2+} signal, see the response to referee #1.

Concerning the detection level for cAMP, this experimental issue was addressed. To this end, we performed experiments in the absence and presence of IBMX, which prevents cAMP breakdown. We, thereby, exclude that a transient increase of cAMP would have escaped detection. We now address the issue of local cAMP signalling in the discussion section.

3. Even better, using patch clamp recordings with GTPγS or GDPβS in the pipette, the authors could significantly strengthen their conclusion, a major one that, if proven, would drastically change how we think about the signaling pathway.

We extended our electrophysiological experiments. The presence of GDPβS did not impair potentiation of CatSper currents by odorants, progesterone, prostaglandins, and 8-Br-cGMP (see also response to referee #1).

4. The Y-axes in several figures (Figs. 1, 3, 5, 7, S1, S4 & S7) should be changed to" ΔF/F0" from "Δ)F/F". To make it easier for the general audience to understand, explain ΔF/F0 when first used (e.g. Fig. 1 legend).

We changed the Y axes accordingly and explain $\Delta F/F_0$ in the legend of Figure 1.

5. In the abstract, consider changing "binding to an extracellular site" to "acting on an extracellular site" because, strictly speaking, a binding is not demonstrated in the studies.

We changed the wording accordingly.

6. page 14, the method section, the solutions used in the patch clamp experiments seem to have a iquid junction potential of ~15 mV, depending on the configuration. Was the potential corrected in the figures and in the Vrev calculation?

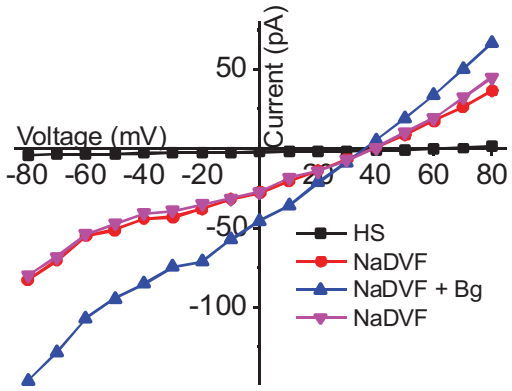
We did not correct for the liquid junction potential, neither in the figures nor in the V_{rev} calculation. This is now mentioned in the material and methods section.

7. in figures where currents from step protocols are shown (Fig. 2A, C; Fig. 6 A, C, E), there appear to be large capacitance currents that could confuse "non-expert" readers. Consider applying a low-pass filter and stating the filter frequency in the method section.

Although it might confuse readers that are not familiar with electrophysiological data, we decided not to remove capacitative transients by filtering. We prefer to show "raw" unprocessed data, whenever possible.

8. figure 2, how easily is the action of bourgeonal reversible? it would be nice to include a washout trace in the figure.

This remark is well founded. The action of all drugs tested was reversible. In patch-clamp experiments, we always check for reversibility of drug action. Because we consider this as "good electrophysiological practice", we did not mention it explicitly. The figure below shows monovalent CatSper currents before (NaDVF, red trace) and after application of bourgeonal (80 µM) (NaDVF + Bg, blue) and after washout of bourgeonal (NaDVF; purple).



9. Figure S2 appears to be inappropriately cited in page 4. Please clarify the conclusion of this figure.

We now cite the figure appropriately. We clarified the conclusion.

Response to referee #3

1. Why was only 4 mM bicarbonate used for cAMP assessments?

Initially, we performed experiments in high bicarbonate (25 mM). Because bourgeonal failed to elevate cAMP levels, we suspected that incubation in high bicarbonate, which stimulates soluble AC, might compromise the detection of a small increase of cAMP. Therefore, we repeated these experiments in 4 mM bicarbonate buffer. The manuscript now includes the data in high and low bicarbonate.

2. An explanation is required why CatSper (no currents activated) is not activated by calcium (page 6).

A hallmark of most if not all Ca^{2+}-permeable channels is that they carry both mono- and divalent ions. The intrapore binding sites for Ca^{2+} usually have a high affinity (nanomolar K_D like for CatSper) and the residence time for Ca^{2+} in the pore is long. Thereby, Ca^{2+} blocks monovalent currents in mixed solutions, a mechanism referred to as "block by permeation". Due to the longer residence time of Ca^{2+} in the pore, Ca^{2+} currents are significantly smaller then monovalent currents. The CatSper channel is no exception to this general finding. In sperm, at 2 mM extracellular Ca^{2+}, divalent CatSper currents are below detection threshold (Kirichok et al. 2006; Lishko et al 2010; Lishko et al 2001; Strünker et al 2011); divalent CatSper currents can only be recorded in solutions containing high (e.g. 50 mM) concentrations of Ca^{2+} or Ba^{2+}. Moreover, many Ca^{2+} channels are inactivated by a negative Ca^{2+}/CaM feedback mechanism. This is another reason why many electrophysiological studies of Ca_v channels use monovalent ions or Ba^{2+} ions.
However, in sperm, only a few Ca^{2+} ions are required to change the $[Ca^{2+}]_i$: in the tiny volume of a sperm flagellum, ~ 3 Ca^{2+} ions equal 1 nM. Thus, opening of a few CatSper channels for a fraction of a second will already change $[Ca^{2+}]_i$ significantly.

3. There is considerable reliance on use of drugs e.g. NNC 55-0396 which are not CatSper specific hence other effects of these drugs needs to be strictly controlled/eliminated.

We fully agree. Therefore, a drug's action should be crosschecked both in Ca^{2+} fluorimetry and electrophysiology. In fact, the pharmacology of all drugs/inhibitors that we tested was complex and we caution against rash interpretations of pharmacological results in sperm (see also response to referee #1).

4. Importantly, the physiological context needs explaining. An explanation/speculation of how the cell may operate in the female tract in a 'soup' of chemicals for a number of days - how is CatSper likely to respond/activate/deactivate? Progesterone, prostaglandins etc are present at the same time in the tract - how does CatSper respond to a combination of reported agonists?

We thank the referee for his suggestion. Accordingly, we have added a small paragraph at the end of the discussion. Inside the female genital tract, several different molecules might act on CatSper either in concert or in a spatially restricted manner. Progesterone and odorants, among several other molecules, have been proposed to attract human sperm. Although the nature of the chemoattractants for human sperm is still controversial, our results indicate that different female factors might target CatSper. It needs to be addressed in future studies whether CatSper activation by different molecules evokes stereotypic motility responses and whether different agonists, in fact, act synergistically or even antagonistically.

5. What is the explanation for figure 1G?

Concerning Figure 1G, we do not know why undecanal suppressed bourgeonal-induced Ca^{2+} responses in studies from Hatt/Spehr's group. Moreover, we find that undecanal itself evokes Ca^{2+} signals in human sperm – even more potently than bourgeonal. When simultaneously delivered with bourgeonal, Ca^{2+} signals are enhanced rather than suppressed, indicating that the action of bourgeonal and undecanal is, in fact, additive. In patch-clamp experiments, we show that both undecanal and bourgeonal directly activate CatSper. Thus, we cannot reconcile our results with previous reports.

6. cAMP is used as a chemoattractant e.g. in Dictyostelium - is this a possible mechanism in humans?

We do not consider this a possible mechanism. In fact, cAMP ($\leq$ 1 mM) does not evoke a Ca^{2+} signal in human sperm. This is now documented in the manuscript (see also response to referee #1).

7. With cAMP assessments as an endpoint the authors report no generation of cAMP with reportedly known agonists. What is the reason for the discrepancy in the results with other previous studies? Is it species differences and/or technique?

The referee points out that our results disagree with several previous reports. However, we note that our results agree with several other reports (e.g. action of adenosine or regulation of sAC).

There might be species differences or subtle technicalities that we are not aware of. However, we should acknowledge a third possibility, namely that results cannot be reconciled because some of them cannot be reproduced and, therefore, should not be considered any further. In fact, there is a substantial body of evidence that human and mouse sperm lack tmACs required for GPCR signalling.

8. The n values need clarification in Fig 4.

The number of experiments is now included.

9. In Supplementary Fig 2 - live AR needs to be clarified e.g. in title.

We removed the term “live cells” because it is misleading and suggests that sperm were alive during the analysis by FACS. In fact, sperm were fixed by incubation in formaldehyde (for details see Jaiswal et al 1999). Staining by PI indicates that sperm were dead even before fixation. Those sperm were excluded from the analysis. We rephrased the figure legend accordingly.

2nd Editorial Decision 05 January 2012

Thank you for sending us your revised manuscript. Our original referees have now seen it again. In general, the referees are now positive about publication of your paper. Still, referee 1 and 2 put forward a few minor issues that still need to be addressed (see below) before we can ultimately accept your manuscript. Furthermore, please reformat the references section to EMBO J. format in the final version of the manuscript (alphabetical order, no numbering - for details, please refer to our 'authors instructions').

Please let us have a suitably amended manuscript as soon as possible. I will then formally accept the manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The authors adequately address most of my concerns. However, the issue of involvement of G protein coupled receptors is not completely resolved. First, when inspecting the response in sperms infused with GDP-beta-S, the current activated by Bg (Fig. 5) and Un (Fig. S8) is about 50% smaller than the signals shown in Fig. 2 in the absence of GDP-beta-S. The authors should show that the average current in the absence and presence of GDP-beta-S are the same. Second, these experiments need a proper control, which show that the GDP-beta-S actually inhibits a GPCR response in the sperm to verify that GDP-beta-S accessed all the sperm cytoplasmic space.

Referee #2

The authors have done a good job addressing the reviewers' comments. Several of their new experiments have strengthened their conclusion. I'm satisfied with the revision.

I have three minor comments.

The currents recorded with GDPbS in the pipettes appear to be smaller than those without GDPbS: ~ 400 pA in Fig. 2B (without GDPbS) and ~ 100 pA in Fig. 5C (with GDPbS). The authors should give the numbers with statistics to test whether GDPbS decreases the current or not. In addition, 0.25 mM of GDPbS was used in the experiment. This concentration is low compared with many of the similar experiments used in neurons where 1 to 2 mM is generally used. This might be a potential problem if diffusion is an issue for the small size of the cells. Did the authors use higher concentration?

Fig. S8 states "Currents were recorded at pHi 7.3 in the absence and presence of intracellular divalent ions and GDPbS (250 μM), respectively." All the panels seem to be from recordings with GDPbS. Please clarify.

p.14 (Discussion) "Membrane-permeable analogues of cyclic nucleotides stimulate Ca2+ entry in mouse and human sperm (Kobori et al, 2000; Ren et al, 2001; Xia et al, 2007; Liu et al, 2007; Xia et al, 2009)." The 5 cited papers only used mouse sperm. A paper using human sperm should be added here, or "mouse and human sperm" should be changed to "mouse sperm".

Referee #3

This is a revised manuscript which contains extra explanatory material and additional experiments. My comments (referee 3) have been sufficiently addressed e.g. bicarbonate concentrations (Fig 3), capacitation status, discussion of in vivo relevance.

The data in this manuscript will significantly stimulate a rethink of some key aspects of the cAMP and calcium signalling events in human spermatozoa. It is likley that this will be a very productive subject in the near future.

2nd Revision - authors' response 13 January 2012

General response to all referees

We would like to thank the referees for their valuable and constructive criticism of the original and revised manuscript.

Response to the referee #1 and #2:

Referee 1: The authors adequately address most of my concerns. However, the issue of involvement of G protein coupled receptors is not completely resolved. First, when inspecting the response in sperms infused with GDP-beta-S, the current activated by Bg (Fig. 5) and Un (Fig. S8) is about 50% smaller than the signals shown in Fig. 2 in the absence of GDP-beta-S. The authors should show that the average current in the absence and presence of GDP-beta-S are the same. Second, these experiments need a proper control, which show that the GDP-beta-S actually inhibits a GPCR response in the sperm to verify that GDP-beta-S accessed all the sperm cytoplasmic space.

Referee 2: The currents recorded with GDPbS in the pipettes appear to be smaller than those without GDPbS: ~ 400 pA in Fig. 2B (without GDPbS) and ~ 100 pA in Fig. 5C (with GDPbS). The authors should give the numbers with statistics to test whether GDPbS decreases the current or not. In addition, 0.25 mM of GDPbS was used in the experiment. This concentration is low compared with many of the similar experiments used in neurons where 1 to 2 mM is generally used. This might be a potential problem if diffusion is an issue for the small size of the cells. Did the authors use higher concentration?

The comments by the referees are well taken. However, it has been shown by Kirichok et al. 2006 (*Nature*) and Lishko et al. 2010 (*Cell*) that the entire sperm cell is rapidly perfused with the pipette solution; perfusion was visualized by a fluorescent dye included in the pipette solution. We performed similar experiments confirming these results. Therefore, we trust that GDPβS accessed the entire cell.

In human sperm, ionic currents regulated by G-proteins have not been identified so far. Thus, for the time being, there is no control experiment available to probe the inhibition of G-proteins by GDPβS.

We agree that, in electrophysiology, GDPβS has been used at concentrations up to 3 mM to prevent G-protein signalling. We did not use millimolar concentrations of GDPβS, because it has been shown that 100 – 500 µM of the drug is sufficient to almost completely abolish G-protein-mediated

activation or inhibition of various ionic currents in neurons and muscle cells. (e.g. Drdla et al. 2009, *Science*; Holz et al. 1986, *Nature*; Aman et al. 2007, *Journal of Pharmacology and Experimental Therapeutics*; Nörenberg et al. 1997, *British Journal of Pharmacology*; Cuevas & Adams 1997, *Journal of Neurophysiology*; So & Kim 2003, *Journal of Smooth Muscle Research*, Wu and Barish 1999, *Journal of Neuroscience*, Gerevich et al. 2005, *European Journal of Pharmacology*). Moreover, in erythrocyte and liver membranes, GDPβS inhibited activation of tmACs by G-proteins with a K_i value of 0.4 μM and 4 μM, respectively (Eckstein 1979, *Journal of Biological Chemistry*). Finally, activation of PLD by G-proteins was also completely abolished by 100-250 μM GDPβS (Stunff et al. 2000, *Biochemical Journal*; Facchinetti et al. 1998, *Journal of Lipid Research*).

Therefore, 250 μM GDPβS should be sufficient to completely abolish activation of CatSper via G-proteins. In fact, bourgeonal enhanced monovalent CatSper currents by 2.3 ± 0.4 and 1.9 ± 0.4 fold in the absence and presence of GDPβS, respectively (Undecanal 2.1 ± 0.2 vs. 1.7 ± 0.2 fold); these results are difficult to reconcile with a pathway that requires activation of G-proteins.

CatSper currents vary considerably between experiments (compare for example Figure 2B and 2D, see also Strünker et al. 2011); therefore, comparison of current amplitudes between individual current traces can be misleading (figures show results of a single experiment). For clarification, we now provide the mean values of CatSper currents at – 60 mV in the absence (96 ± 58 pA, range 30 - 236 pA) and presence (65 ± 27 pA, range 27 - 141 pA) of GDPβS along with the mean relative current increase stimulated by the odorants.

We rephrased the GDPβS paragraph accordingly.

Response to referee # 2

Fig. S8 states "Currents were recorded at pHi 7.3 in the absence and presence of intracellular divalent ions and GDPbS (250 μM), respectively." All the panels seem to be from recordings with GDPbS. Please clarify.

The figure legend is indeed misleading. As the referee points out, all experiments were performed in the presence of GDPβS. We rephrased the legend accordingly.

p.14 (Discussion) "Membrane-permeable analogues of cyclic nucleotides stimulate Ca2+ entry in mouse and human sperm (Kobori et al, 2000; Ren et al, 2001; Xia et al, 2007; Liu et al, 2007; Xia et al, 2009)." The 5 cited papers only used mouse sperm. A paper using human sperm should be added here, or "mouse and human sperm" should be changed to "mouse sperm".

We agree. However, in the results section, we cited Machado-Oliveira et al. (2008) for the action of 8-Br-cNMPs on human sperm. We now include this reference also in the discussion section.

